Applied and Environmental Microbiology, Dec. 2010, p. 7903–7909 Vol. 76, No. 24
0099-2240/10/$12.00 doi:10.1128/AEM.01252-10

Effect of Sulfadiazine-Contaminated Pig Manure on the Abundances of Genes and Transcripts Involved in Nitrogen Transformation in the Root-Rhizosphere Complexes of Maize and Clover▽†

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Received 27 May 2010/Accepted 26 September 2010

The antibiotic sulfadiazine (SDZ) can enter the environment by application of manure from antibiotic-treated animals to arable soil. Because antibiotics are explicitly designed to target microorganisms, they likely affect microbes in the soil ecosystem, compromising important soil functions and disturbing processes in nutrient cycles. In a greenhouse experiment, we investigated the impact of sulfadiazine-contaminated pig manure on functional microbial communities involved in key processes of the nitrogen cycle in the root-rhizosphere complexes (RRCs) of maize (*Zea mays*) and clover (*Trifolium alexandrinum*). At both the gene and transcript level, we performed real-time PCR using *nifH*, *amoA* (in both ammonia-oxidizing bacteria and archaea), *nirK*, *nirS*, and *nosZ* as molecular markers for nitrogen fixation, nitrification, and denitrification. Sampling was performed 10, 20, and 30 days after the application. SDZ affected the abundance pattern of all investigated genes in the RRCs of both plant species (with stronger effects in the RRC of clover) 20 and 30 days after the addition. Surprisingly, effects on the transcript level were less pronounced, which might indicate that parts of the investigated functional groups were tolerant or resistant against SDZ or, as in the case of *nifH* and clover, have been protected by the nodules.

Antibiotics have been used in animal husbandry worldwide to treat infectious diseases. Sulfonamides, including sulfadiazine (SDZ), belong to one of the major groups of veterinary drugs and are mainly used in pigs (10). Sulfonamides are poorly adsorbed in the animal gut; consequently, they are excreted unchanged in urine and feces (1, 12, 16, 26) and reach the soil ecosystem via manuring. Sulfonamides have been characterized as broad-spectrum antibiotics with a bacteriostatic mode of action based on inhibition of folic acid metabolism (9). Therefore, sulfonamides in the environment may impact soil health by changing microbial activity patterns and the kinetics of important turnover processes (6, 25, 48). Although changes in turnover rates have been reported for some microbial processes (e.g., see reference 24), a number of studies in the last decade have shown that the influence of sulfonamides on microbes and their metabolic performance in bulk soil is relatively low (42). These findings have been explained by (i) the large microbial diversity in bulk soil systems and by possible mechanisms of functional redundancy (37), (ii) the relatively low activity of microbes in bulk soil that are nearing the dormancy state (40) in which microbes are not affected by sulfonamides, and (iii) the development of resistant populations by horizontal gene transfer (20).

The impact of sulfonamides on microbial turnover processes may be more pronounced in highly active microbial communities (e.g., in the rhizosphere) than in inactive or dormant microorganisms in bulk soil. The rhizosphere is defined as the soil that is influenced by the plant root system, which strongly affects the surrounding soil chemistry through nutrient depletion, acidification, and the secretion of organic substances (53). Due to the organic compounds they release, plant roots provide suitable ecological niches for microbial growth and activity (3). Furthermore, several studies have indicated changed or even reduced diversity patterns in the rhizospheres compared to bulk soils (29). Therefore, it can be postulated that the effects of antibiotic-contaminated manure on microbes in rhizosphere soil might differ from that in bulk soil. Moreover, the extent of the antibiotic effect together with the manure effect is difficult to predict due to the complexity of plant-microbe interactions in the rhizosphere.

As rhizosphere microbial communities exert strong effects on plant quality (53), the questions of whether and how antibiotics in soil alter microbial activities in the rhizosphere are of interest not only for basic research and ecotoxicology but also for farmers and plant breeders. Supplying plants with nitrogen is of key importance for yield and plant health in agricultural ecosystems.

The aim of this study was to investigate the effects of the antibiotic sulfadiazine in combination with pig manure (PM)

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 22 October 2010.

on the functional microbial biomass involved in nitrogen (N) cycling in the root-rhizosphere complex (RRC) of two different agricultural crops, a C_4 plant (maize [*Zea mays*]) and a legume (berseem clover [*Trifolium alexandrinum*]). As it is well accepted that plant species are a dominant factor influencing the composition of the rhizosphere microbial communities (54), we chose for the cultivation of the two different crops one typical arable soil. SDZ and manure concentrations applied were in accordance with agricultural practice in Germany (48). Ten, 20, and 30 days after application, we analyzed the abundance of functional genes (representing the genetic potential for the corresponding pathways) catalyzing key steps of nitrification, denitrification, and nitrogen fixation as well as transcripts of the same marker genes (representing the expression level of the enzymes under study) in order to reconstruct major parts of the nitrogen cycle in the RRC.

We hypothesized that nitrogen turnover processes in the RRC would be affected by the application of SDZ and that the changes would consequently influence plant growth, especially in the case of the legume where the symbiosis between plant and microbes is a major determinant of plant health. Furthermore, we assumed that the abundance patterns of transcripts would be more affected than the gene copy numbers due to the bacteriostatic mode of action of the antibiotic.

MATERIALS AND METHODS

Experimental design. A silt loam (Orthic Luvisol) from the A_p horizon (0 to 40 cm) of an agricultural field located near Merzenhausen, Germany (50° 56′ 3″ N, 6° 17′ 31″ E; see Table S1 in the supplemental material) that had not been previously fertilized with manure was used for the greenhouse experiment. The experiment was conducted in a randomized block design with four independent replicates per treatment and sampling time point. Polypropylene tubes (height, 70 cm; diameter, 15 cm) containing 14 kg of air-dried soil (sieved at 4 mm) were used. After an equilibration phase of 14 days at 20°C and 50% maximum water holding capacity of the soil, three seeds of maize (*Zea mays*) and 30 seeds of berseem clover (*Trifolium alexandrinum*) were sown per pot. Two weeks after germination, 250 ml each of pig manure (PM) and pig manure contaminated with sulfadiazine (PMSDZ) were applied per tube to the soil surface, resulting in final concentrations of 7.4 mg nitrogen kg^{-1} soil and 20 mg SDZ kg^{-1} soil (in the upper 20 cm). The plants were subjected to a photoperiod of 15 h of light and 9 h of darkness with a constant soil water content. Sampling was performed 10, 20, and 30 days after manure application; each of the four independent replicates was treated separately. A composite sample of roots with the attached soil was taken from the upper 20 cm of each tube. After the roots were vigorously shaken, the roots and attached soil were treated as one compartment called the root-rhizosphere complex (RRC). One part of the RRC was immediately snap-frozen in liquid nitrogen and stored at −80°C for nucleic acid extraction; the other part was directly extracted with 0.01 M $CaCl_2$ for the determination of water-extractable organic carbon (WEOC), water-extractable organic nitrogen (WEON), ammonium-N (NH_4^+-N), and nitrate-N (NO_3^--N) concentrations. Samples of homogenized bulk soil were frozen at −20°C until used for the quantification of SDZ and its metabolites.

Sulfadiazine and metabolites in bulk soil samples. The totally desorbable and hence potentially bioavailable SDZ fraction and its metabolites, *N*-acetyl-SDZ and 4-hydroxy-SDZ, were isolated by a sequential extraction procedure using 0.01 M $CaCl_2$ (soil/solution ratio of 1:2.5) followed by an extraction step with methanol (MeOH) (soil/solution ratio of 1:2.5) (17). Separation and detection of extracted sulfadiazine and its metabolites were carried out using a Shimadzu Prominence LC20 high-performance liquid chromatography (HPLC) system. The stationary phase consisted of a SunFire C_{18} column (3.0- by 100 mm; 3.5-μm particle size; Waters, Germany). The injected volume (10 μl) was mobilized at a flow rate of 300 μl min^{-1} in a gradient program by phase A (0.1% HCOOH in water) and phase B (0.1% HCOOH in MeOH). HPLC-separated fragment ions of sulfadiazine and metabolites were captured using an API 3200 mass spectrometer (Applied Biosystems, Germany). The data were analyzed using the Analyst 1.4.2 application (Applied Biosystems, Germany) with a minimum signal-to-noise ratio (SNR) of 10:1. The limit of detection was determined by the method of Antignac et al. (2) and was in the range of 0.2 ng SDZ, 1 ng hydroxy-SDZ, and 5 ng *N*-acetyl-SDZ g^{-1} soil, respectively.

Nitrogen and carbon content in plants. The total green plant biomass was dried at 65°C for 48 h, ball milled (Retsch MM2; Retsch GmbH, Germany) and transferred into tin capsules (5- by 3.5 mm; HEKAtech GmbH, Germany). Total carbon and nitrogen contents were determined using the elemental analyzer Euro-EA (Eurovector, Italy) (32).

Water-extractable nitrogen and carbon fractions in the RRC. Samples consisting of 5 g RRC were shaken overhead for 45 min in 25 ml of 0.01 M $CaCl_2$. After filtration, water-extractable total nitrogen and organic carbon were measured using a total organic carbon (TOC) analyzer (DIMA-TOC 100; DIMATEC, Germany) equipped with a total bound nitrogen (TNb) module. A continuous-flow analyzer (SA 20/40; Skalar Analytical, Netherlands) was used to determine ammonium-N and nitrate-N. Water-extractable organic nitrogen was calculated as the difference between total nitrogen and ammonium plus nitrate.

DNA and RNA coextraction and separation. DNA and RNA were coextracted from 0.5 g of RRC by the method described by Griffiths et al. (13). Extraction was performed with Precellys-Keramik kit lysing tubes (Peqlab Biotechnologie GmbH, Germany) in combination with the Bertin Precellys 24 bead beating system (Bertin Technologie, France). DNA and RNA were separated using the AllPrep DNA/RNA minikit (Qiagen, Germany) according to the manufacturer's instructions. DNA and RNA yield and purity were measured with a microvolume fluorospectrometer (NanoDrop Technologies, DE). Contamination of RNA samples with coextracted DNA was excluded by PCR assays targeting the 16S rRNA genes using the universal primers 341F (5′-CTGCTGCCTCCCGTAG-3′) and 1401R (5′-CGGTGTGTACAAGACCC-3′) (36).

Single-stranded cDNA synthesis from total RNA. Samples of 2.5 μg total RNA were converted into single-stranded cDNA by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Germany) according to the manufacturer's instructions. cDNA yield and purity were measured using the microvolume fluorospectrometer.

Abundance of functional genes and their transcripts. Quantitative PCR (qPCR) was used to determine the abundance of functional communities involved in the nitrogen cycle and their activities by targeting genes and their corresponding transcripts encoding key enzymes of nitrogen fixation (*nifH* encoding nitrogenase), ammonia oxidation (*amoA* encoding ammonia monooxygenase) in both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), and denitrification (*nirK*, *nirS*, and *nosZ* encoding cytochrome cd_1, copper nitrite reductases, and nitrous oxide reductase, respectively). Absolute quantification of all investigated target genes was carried out in 25-μl samples in triplicate on the ABI Prism 7300 cycler (Applied Biosystems, Germany) with the following reagents: bovine serum albumin (Sigma-Aldrich, Germany), primers (Table 1) (Metabion, Germany), dimethyl sulfoxide (Sigma, Germany), and Power SYBR green PCR master mix (Applied Biosystems, Germany) (21). All PCR runs started with an initial enzyme activation step performed at 95°C for 10 min, but the subsequent thermal profiles differed from gene to gene as indicated in Table 1. The specificity of the amplification products was confirmed by melting-curve analysis. No template controls gave a null or negligible value. To avoid inhibitory effects on quantitative PCR, samples were diluted 10-fold based on results from a previous experiment (data not shown). Dilution series of plasmid DNA with cloned bacterial *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* genes and archaeal *amoA* functional gene fragments were used to generate standard curves ranging from 10^0 to 10^5 gene copies μl^{-1} for cDNA quantification and from 10^1 to 10^6 gene copies μl^{-1} for DNA quantification with efficiencies ranging from 97 to 100%.

Statistical analysis. Prior to analysis, DNA and cDNA abundance data were log transformed (ln) to achieve normal distribution. Data were analyzed by two-way analysis of variance (ANOVA) with treatment (PM, PMSDZ) and time (10, 20, and 30 days) as independent factors. Homogeneity of the variances was checked by the Levene test. The significance level was set to $\alpha = 0.05$. Furthermore, independent *t* tests were used to test for a significant difference between the two treatments at a given time point with significance level corrected by the Šidák's equation to $\alpha = 1 - (1 - 0.05)^{1/3} = 0.017$. Statistical tests were calculated with SPSS 11.5 (SPSS, Inc., IL).

RESULTS

SDZ concentrations in bulk soil samples. At all sampling time points, the concentrations of $CaCl_2$/methanol-extractable sulfadiazine (SDZ), *N*-acetyl-SDZ, and 4-hydroxy-SDZ in bulk

TABLE 1. Primers and thermal profiles used for real-time PCR quantification of different functional genes

Target gene[a]	Primer set[b]	Reference	Thermal profile	No. of cycles	Amplicon size (bp)
nifH	*nifH*-F-Rosch	39	45 s at 95°C, 45 s at 55°C, and 45 s at 72°C	40	458
	nifH-R-Rosch	39			
AOB *amoA*	*amoA*-1F	41	60 s at 94°C, 60 s at 60°C, and 60 s at 72°C	40	500
	amoA-2R	41			
AOA *amoA*	19F	27	45 s at 94°C, 45 s at 50°C, and 45 s at 72°C	40	624
	CrenamoA616r48x	43			
nirK	*nirK*-876	18	15 s at 95°C, 30 s at 63°C to 58°C, and 30 s at 72°C	6 td[c]	164
	nirK-5R	7		40	
nirS	*nirS*-cd3af	35	60 s at 94°C, 60 s at 57°C, and 60 s at 72°C	40	413
	nirS-R3cd	50			
nosZ	nosZ2F	19	15 s at 95°C, 30 s at 65°C to 60°C, and 30 s at 72°C	6 td	267
	nosZ2R	19		40	

[a] Abbreviations: AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea.
[b] The forward (F) and reverse (R) primers are indicated.
[c] td, touchdown.

soil were below 0.2, 1, and 5 ng g^{-1}, respectively (data not shown).

Nitrogen and carbon content of the plants. For both plant species, the yield of the green biomass was not influenced by the presence of SDZ in the manure applied at the sampling time points. In the clover biomass, increased nitrogen content was found compared to maize and decreased N content was observed with pig manure contaminated with sulfadiazine (PMSDZ) treatment (see Tables S2 and S3 in the supplemental material). A significant treatment effect on the carbon content of the maize plants was revealed by slightly higher carbon concentration in the PMSDZ treatment after 10 days (see Tables S2 and S4 in the supplemental material).

Nitrogen and carbon content in the RRC. The application of SDZ had no significant effect on the amount of water-extractable organic nitrogen (WEON) and carbon in the root-rhizosphere complex (RRC) of either plant type (see Table S3 in the supplemental material). However, plant species- and time-dependent differences, i.e., larger amounts of WEON in the RRC of the legume, were detected (Fig. 1; see Table S2 in the supplemental material). Differences in ammonium and nitrate concentrations between the treatments were observed; in the RRC of clover 10 days after application, there were lower ammonium concentrations in the PMSDZ treatment (20 μg NH_4^+-N g^{-1}) than in the PM treatment (50 μg NH_4^+-N g^{-1}) ($P = 0.044$). In the RRC of maize 30 days after application of the different types of manure, the ammonium concentration was higher in the PMSDZ treatment than in the PM treatment ($P = 0.026$).

Quantification of functional genes and their corresponding transcripts. For all genes and transcripts, clear variations over time were visible in both plant species.

In clover RRC, SDZ contamination of the manure significantly reduced *nifH*, ammonia-oxidizing bacteria (AOB) *amoA*, *nirK*, *nirS*, and *nosZ* gene abundance patterns 20 days after application (Fig. 2A and Table 2). At this time point, the difference in copy numbers between the treatments PM and PMSDZ reached up to 2 orders of magnitude. AOB *amoA*, *nirK*, *nirS*, and *nosZ* copy numbers remained lower in the PMSDZ treatment at day 30. In contrast, ammonia-oxidizing archaea (AOA) *amoA* gene abundance was not influenced at any sampling time point by the SDZ-treated manure. Similar to the observations at the DNA level, AOA *amoA* transcripts did not respond to the contaminated manure. As expected, the reduced number of genes involved in denitrification (*nirS*, *nirK*, and *nosZ*) and nitrification (AOB *amoA*) in the PMSDZ treatment resulted in a reduced number of transcripts. Moreover,

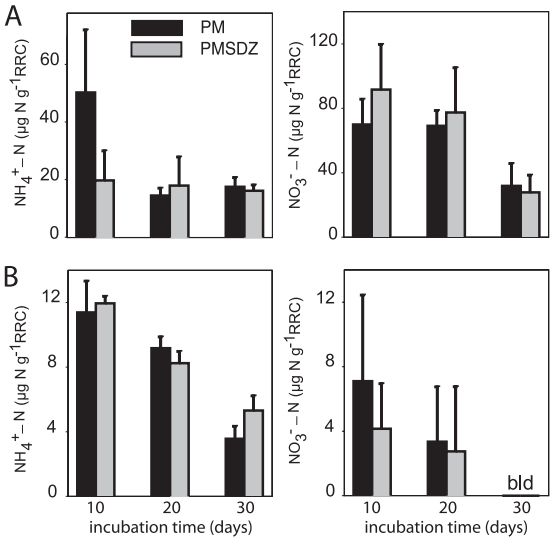

FIG. 1. Ammonium and nitrate concentrations in the root-rhizosphere complex of clover (A) and maize (B) after the addition of pig manure (PM) or pig manure plus sulfadiazine (PMSDZ) at three different time points (10, 20, and 30 days) after application. Error bars represent standard deviations of means ($n = 4$). Abbreviations: RRC, root-rhizosphere complex; bld, below limit of detection.

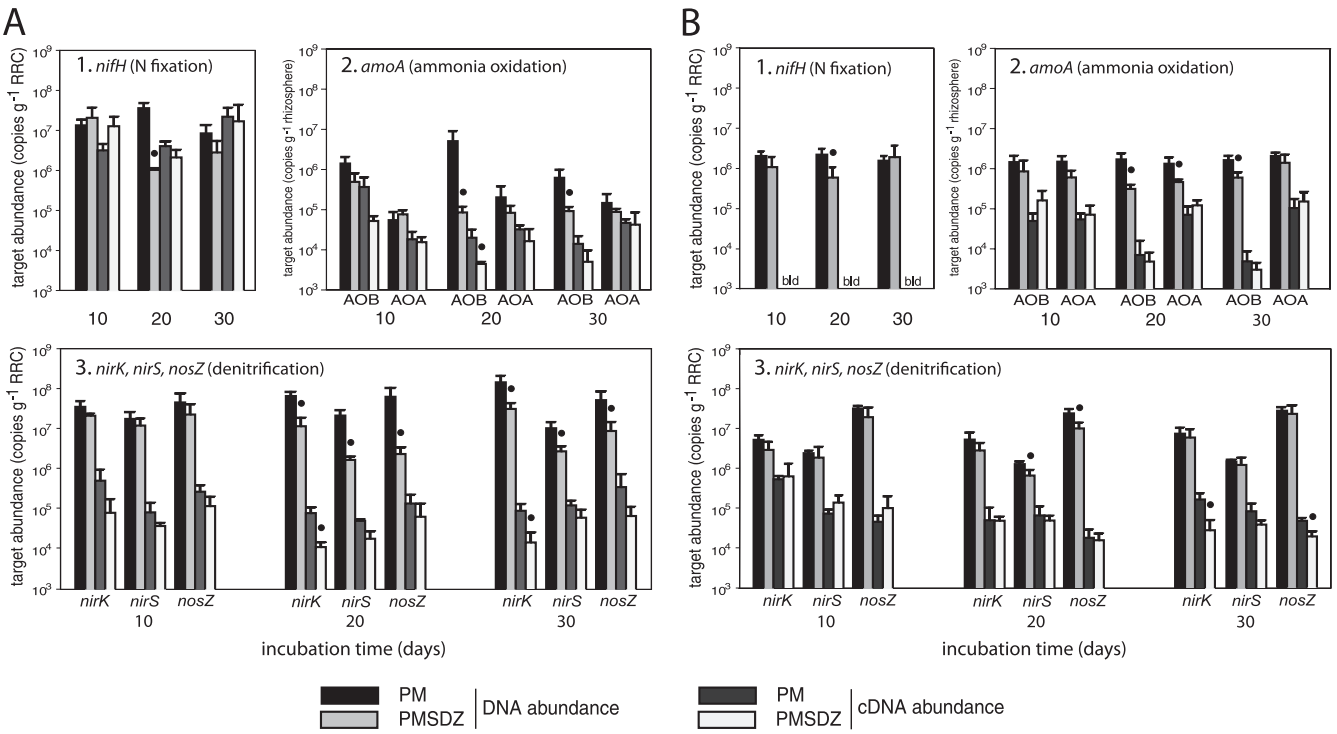

FIG. 2. Quantification of functional gene and transcript copies involved in nitrogen cycling (*nifH*, AOB *amoA*, AOA *amoA*, *nirK*, *nirS*, and *nosZ*) in the root-rhizosphere complex of clover (A) and maize (B) after the addition of pig manure (PM) or pig manure plus sulfadiazine (PMSDZ) at three different time points (10, 20, and 30 days) after application. Significant differences between the two treatments at a particular time point are indicated by solid black circles ($P < 0.017$). Error bars represent standard deviations of means ($n = 4$). Abbreviations: RRC, root-rhizosphere complex; bld, below limit of detection; AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria.

we observed a reduced number of transcripts for AOB *amoA* 10 days after application ($P = 0.023$) and lower abundance values for *nirK* transcripts ($P = 0.032$) 30 days after application of the PMSDZ compared to the control PM treatment. However, despite reduced gene abundance, *nifH* transcripts were not affected by PMSDZ.

In maize RRC, *nifH*, AOA and AOB *amoA*, *nirK*, *nirS*, and *nosZ* gene levels were significantly decreased by PMSDZ treatment, notably 20 days after application (in the range of 0.5 order of magnitude; Fig. 2B and Table 2). Whereas most of the investigated genes in the PMSDZ-treated samples exhibited recovery at a later time point, AOB *amoA* gene copy numbers remained low compared to the corresponding PM-treated samples 30 days after application. The abundance of transcripts for *nirK*, *nirS*, and *nosZ* in maize RCC was not affected by PMSDZ at the early sampling time points. However, 30 days after application, lower levels of *nirK*, *nosZ*, and *nirS* transcript copy numbers were observed in the PMSDZ treatment (Fig. 2B). Abundance patterns of *amoA* (AOB and AOA) transcripts were not affected by the treatment (Table 2), whereas the number of *nifH* transcripts was below the detection limit at all sampling time points.

TABLE 2. Statistical evaluation of gene and transcript abundance by two-way ANOVA

Plant and factor	*P* value[a]											
	nifH		AOB *amoA*		AOA *amoA*		*nirK*		*nirS*		*nosZ*	
	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA	DNA	cDNA
Clover												
Treatment	**0.000**	0.764	**0.000**	**0.000**	0.553	0.094	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**	**0.007**
Time	**0.002**	**0.045**	**0.002**	**0.000**	0.165	0.117	**0.001**	**0.002**	**0.000**	**0.003**	0.055	0.222
Treatment × time	**0.000**	**0.000**	**0.001**	0.244	0.250	0.439	**0.012**	0.984	**0.000**	0.576	**0.009**	0.777
Maize												
Treatment	**0.006**		**0.000**	0.408	**0.001**	0.159	**0.028**	0.263	**0.020**	0.616	**0.006**	0.426
Time	0.493		0.487	**0.000**	**0.014**	0.266	0.174	**0.000**	**0.047**	**0.035**	0.241	**0.008**
Treatment × time	0.120		0.306	0.265	0.605	0.675	0.828	**0.034**	0.747	0.059	0.551	0.201

[a] The *P* values show the impact of the manure treatments and time on functional genes and their corresponding transcripts involved in nitrogen turnover. Boldface values indicate significant effects ($P < 0.05$).

DISCUSSION

The objective of this study was to assess the impact of a single application of SDZ-contaminated manure on the functional biomass involved in major nitrogen turnover processes in the RRCs of two different crops. By targeting marker genes for nitrification, denitrification, and nitrogen fixation, we quantified the functional communities and functionally redundant populations (37, 38, 43) on both the DNA and RNA level to comprehend the effect of SDZ on the genetic potential and activity status. Although molecular methods have been shown to be suitable tools to improve our understanding of microbial community structure and function in soils, they are accompanied by drawbacks, such as biased extractions of nucleic acids from soils. Therefore, it is difficult to compare results that are based on the same extraction protocol but derive from samples of different soil type and texture, as extraction efficiencies of DNA and RNA might differ. In addition, primer selection for PCR as a possible cause of bias should be kept in mind, as not all environmental sequences of the targeted genes might be detected due to the limited number of species used for primer development. For example, the primers used to target *nosZ* in this study are probably specific only for the nitrous oxide reductase gene from Gram-negative bacteria (19), and thus, the response of the functional biomass to the antibiotic could be underestimated. Moreover, metagenomic analysis has demonstrated in the past few years that protein families can cover a much broader sequence diversity than that usually captured (5, 51, 52).

Differences in DNA and RNA levels. As much care was taken to use the same protocol for DNA and RNA extraction, a comparison on the effect level was possible in this study. The quantification of both genes and transcripts revealed, in contrast to our assumption based on the bacteriostatic mode of action of SDZ, significant impacts on the gene level but to a lower extent on the transcript level. Presumably, microbial subpopulations intrinsically able to cope with the antibiotic stressor could have taken advantage of the altered competitive environment and maintained nitrogen turnover. Whether a significant community change took place under the influence of SDZ remains to be clarified.

Delayed microbial response. Ten days after application of PMSDZ, no $CaCl_2$/methanol-extractable SDZ was detected in bulk soil samples. It has been shown that the potentially bioavailable fractions of SDZ quickly decline in bulk soil (17), because sulfonamides have a large potential for rapid adsorption into the soil matrix and manure constituents (23, 49). In both maize and clover RRCs, we observed a time lag between the application of SDZ and its effect on the functional microbial biomass (Fig. 2). Significant effects of the antibiotic-contaminated manure on gene abundance levels were observed 20 and 30 days after application, whereas the concentration of bioavailable SDZ in the surrounding bulk soil had declined below the detection limit. Such a time lag may be related to the slow generation times of microbes in soil and the bacteriostatic action of sulfonamide. However, it must be taken into account that the fate of SDZ in the rhizosphere may differ from that in bulk soil due to differences in chemical, physical, and microbial properties. Decreased pH values by up to two pH units in the rhizosphere compared to bulk soil (8) may affect SDZ bioavailability. This has been demonstrated for other xenobiotics; a faster degradation of selected pesticide residues was observed in rhizosphere soil than in bulk soil (11).

Rhizosphere effects of different plant types. The differences in gene abundance patterns between the two treatments were more pronounced and long-lasting in the RRC of clover than in the RRC of maize. Presumably, differences in quality and quantity of root exudates and in root morphology known to shape microbial communities and to form the basis for microbial activity in the rhizosphere might have contributed to the different effects observed (22, 30). Whereas maize may have provided primarily recalcitrant organic carbon from decaying root material to the microflora of its RRC (44), clover roots might have excreted more readily available organic compounds (15), resulting in an increase in microbial biomass and activity in this RRC. In contrast, AOA, which might exhibit a rather oligotrophic lifestyle (31), could have been outcompeted by the faster-growing microorganisms in the clover RRC, as indicated by the reduced numbers of AOA *amoA* gene copies found in the clover RRC compared to the maize RRC. As dormancy or reduced activity results in reduced susceptibility to SDZ, the bacteriostatic antibiotic might have found fewer targets (4, 28), thus explaining the differences observed in the effect of SDZ when both plant species were compared.

***nifH*.** Legume roots exude various flavonoid and isoflavonoid molecules that are known to induce development of symbiotic interactions between the plant and nitrogen-fixing alphaproteobacteria within root nodules (46). This is consistent with our results showing explicitly higher *nifH* gene expression and, to a lower extent, higher *nifH* gene abundance in the clover RRC, which includes nodules, compared to the maize RRC. We postulated that legume growth would be more affected by SDZ, as legumes need a symbiotic partner for an optimal supply of nitrogen. Despite a considerable decrease in *nifH* gene abundance in the clover RRC 20 days after application of the SDZ-contaminated manure, the abundance of transcripts was not significantly affected by the antibiotic, which might be the reason for similar plant quality and yield in both treatments. It is possible that the active nitrogen-fixing bacteria within the root nodules are protected from the antibiotic and therefore are not affected. However, it must be noted that external nitrogen was introduced to the soil during manure application; thus, nitrogen provided by nitrogen fixers was not needed to maintain a high plant yield.

***amoA* (AOB and AOA).** In the RRC of clover, the antibiotic abolished the increase of the ammonia-oxidizing bacterial population between day 10 and 20 in response to the manure application (PM). Similar results were shown by Schauss and coworkers (43) in bulk soil, although the effect of SDZ was less pronounced. Moreover, 20 and 30 days after application of the manure, the AOB abundance patterns in both plant RRCs were significantly reduced in the PMSDZ treatment, indicating a sustainable reduction in nitrification potential (Fig. 2). Lower ammonium values were measured in the PMSDZ treatment at the 10-day time point that might be related to an overall inhibition of nitrogen mineralization by the antibiotic. This relative ammonia depletion clearly induced lower bacterial *amoA* transcripts in comparison to the PM treatment and consequently resulted in a lower availability of nitrite, which in turn may have affected denitrifiers harboring the *nirK* gene and a lower

transcript abundance of the corresponding gene. Genes and transcripts of archaeal ammonia oxidizers were not affected by SDZ in the RRC of clover, possibly due to their lower abundance compared to AOB. In contrast, in the maize RRC, AOA were as abundant as AOB during the sampling period and although AOA were significantly influenced by SDZ, they were affected to a lower extent than AOB were, indicating a reduced susceptibility of AOA toward the antibiotic. Thus, the functional redundancy between AOB and AOA under antibiotic stress described by Schauss and coworkers (43) for bulk soil could also be a mode of action in the RRC.

***nirK*, *nirS*, and *nosZ*.** As indicated by decreased copy numbers of all three genes involved in denitrification, the denitrification potential in both plant RRCs was reduced 20 days after application of PMSDZ and remained low in the clover RRC even until day 30. This is in contrast to results obtained in bulk soil where denitrifiers were only slightly affected by SDZ (unpublished data) and underlines the assumption of more pronounced antibiotic effects on highly active microbial communities living in hot spots like the RRC.

It has been shown in several studies that microbes harboring the *nirK* gene form the major part of nitrite reducers in different rhizospheres (14, 21) and show increased activity compared to bacteria harboring the *nirS* gene (45). In our study, we confirmed higher *nirK* gene copy numbers than *nirS* gene copy numbers in both treatments at all sampling time points and observed that *nirK*-harboring microbes were more affected by SDZ than *nirS*-denitrifying bacteria. In addition to the differences in activity between *nirS*- and *nirK*-harboring microbes in the RRC samples, which may explain the differences in response to SDZ, an indirect antibiotic effect could have contributed to the reduced abundances of *nirK* (and *nosZ*) transcripts via impaired microbial respiration activity in the RRC, hence higher oxygen levels and consequently inhibited gene expression in the PMSDZ treatment. Furthermore, the uptake of SDZ may vary between different functional populations. Recently, Zarfl and coworkers (55) described a mechanistic model explaining substance-specific and pH-dependent antibiotic effects. In this model, they assumed that differences in the accumulation and speciation of sulfonamides in bacteria are due to different abilities of bacteria to regulate their internal pH value. Tappe and colleagues (47) examined the influence of diverse sulfonamides, including SDZ, on bacterial growth at different pH values and concluded that a possible impact on the microbial population in soil could strongly depend on the method by which bacteria regulate their internal pH value. This could explain the dissimilarities observed in the responses of the denitrifiers in the RRC.

Conclusion. This greenhouse study revealed that a single application of manure contaminated with the antibiotic sulfadiazine has a lasting impact on the functional microbial biomass involved in nitrogen cycling on both the gene and transcript levels in the RRCs of different plants of agricultural importance. However, the data presented are based on constant climatic conditions, and the role of environmental factors like drought periods on the antibiotic effect has been excluded. Besides, influences of the antibiotic in combination with other agricultural management tools, e.g., the application of pesticides (mainly the role of fungicides), have to be assessed before the results can be transferred into practice. Finally, in this study, a single application of SDZ-contaminated manure was performed, but under field conditions manure (possibly contaminated with antibiotics) is applied several times during the vegetation period and consequently, the microbial communities might adapt to the antibiotic stressor.

Furthermore, it remains to be studied how microbial diversity patterns are affected by antibiotics. It might be assumed that for denitrification this issue is not of such high relevance because many soil prokaryotes are able to use nitrate and nitrite as terminal electron acceptors when oxygen is lacking. In contrast, for other processes like nitrogen fixation and ammonia oxidation, which can only be performed by a limited number of soil microbes, this topic seems of interest. In addition, the question of how the antibiotic might influence the organic nitrogen cycle, mainly the process of nitrogen mineralization, needs to be investigated in further experiments.

ACKNOWLEDGMENT

This work was part of the German Research Foundation (DFG) research group (FOR 566) "Veterinary Medicines in Soils: Basic Research for Risk Analysis."

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